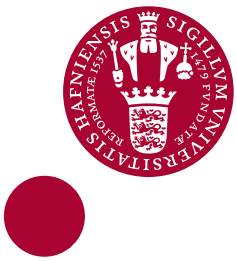

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Publication date:
2010

Document version
Publisher's PDF, also known as Version of record

4th Conference on Physiology of Yeast and Filamentous Fungi

PROGRAMME & ABSTRACT BOOK

1 - 4 June 2010, Rotterdam
The Netherlands

DEPARTMENT OF FOOD SCIENCE
FACULTY OF LIFE SCIENCES
UNIVERSITY OF COPENHAGEN

Production of alcohol-based quorum sensing molecules and their role in biofilm formation and sliding motility of the dairy-important yeast *Debaryomyces hansenii*

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Aim

The aim of the present study was to examine aromatic alcohols including phenylethanol, tyrosol and tryptophol and the sesquiterpene farnesol for their potential quorum sensing effects in the dairy-important yeast *Debaryomyces hansenii*

Introduction

Many yeasts are dimorphic, which means that they are able undergo a transition from a unicellular yeast form to a filamentous form or vice-versa. The yeast-to-mycelium transition can be induced by environmental factors e.g. temperatures below or over the optimal range, limited availability of oxygen, high osmolality pH or substrate limitation including nitrogen starvation. In addition, quorum sensing (the process by which microorganisms communicate by signalling molecules in a cell density dependent manner) has reported to be involved in the morphogenesis of yeasts. The sesquiterpene farnesol has been found to be a signalling molecule inhibiting both the yeast-to-hyphal shift and biofilm formation of *Candida albicans*. In addition, the aromatic alcohol tyrosol has been shown due to its promotion of hyphal development to shorten lag phase time of *C. albicans*. Furthermore, the aromatic alcohols phenylethanol and tryptophol have been found to be signalling molecules in *Saccharomyces cerevisiae*, where they stimulate pseudohyphal growth. Furthermore, phenylethanol was found to stimulate invasive growth. Addition of tryptophol resulted in an even greater invasive growth, whereas tryptophol alone did not have an effect on invasive growth. *C. albicans* does also produce phenylethanol and tryptophol, but if a function, it is different, from what is observed in *S. cerevisiae*.

Material and methods

Three strains of *Debaryomyces hansenii* (type strain CBS767 and the dairy isolates D18335 and MD02 were included. *Candida albicans* (CBS8758) and *Saccharomyces cerevisiae* (CBS1171) were included as reference strains. At standard conditions, yeast cultures were grown in yeast nitrogen base (YNB) without amino acids and ammonium sulfate supplemented with 2% glucose and 5 mM L-Proline (pH 4.3) at 25°C with shaking at 120 rpm. At appropriate time intervals, cell-free supernatants were prepared and analysed by their content of phenylethanol, tyrosol and tryptophol by ESI$^+$ with the triple quadrupole operating in multiple reaction monitoring mode with two transitions per compound. Quantification was done using $^2H_5$-1-phenylethanol as internal standard (10 ng/mL) and using peak areas as response factor relative to $^2H_5$-1-phenylethanol. Farnesol was determined in a similar fashion applying farnesal as internal standard.

Biofilm formation was measured by the crystal violet method. Sliding motility was measured by inoculation of yeast cells in the centre of YPD plates with 0.3% agar, and growth was followed for 14 days. To induce pseudohyphal growth, yeast cells were restreaked on SLAD agar. Plates were incubated at 25°C for 10 days. Colony morphology photographed under a microscope.

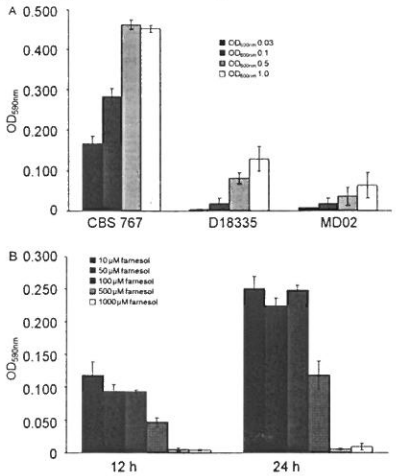

Figure 2. A: Biofilm formation of *D. hansenii* (CBS767, D18335 and MD02) after 24 h of incubation. B: Influence of farnesol on biofilm formation of *D. hansenii* (CBS767).

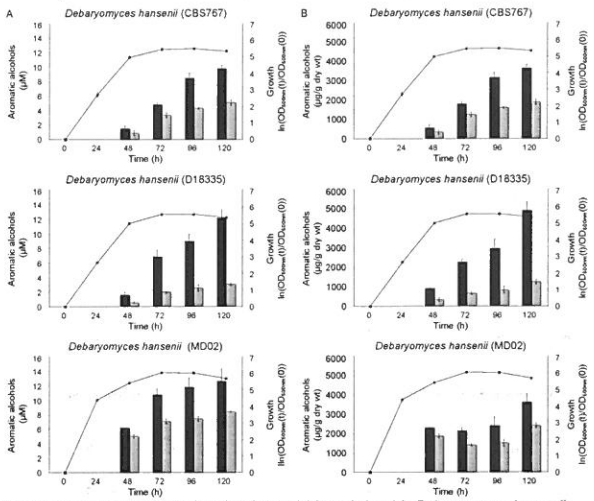

Figure 1. Production of phenylethanol and tyrosol (shown in bars) in *Debaryomyces hansenii* (CBS767, D18335 and MD02) at standard conditions. ■ phenylethanol and ■ tyrosol. Furthermore, growth curves are shown. ■ Growth ($\ln(OD_{600nm}(t)/OD_{600nm}(0))$, t refers to the time.

Table 1. Effects of environmental conditions on phenylethanol and tyrosol production in *Debaryomyces hansenii* (CBS767). Fold inductions/repressions are based on alcohols concentrations in µM determined after 120 h of fermentation.

	Fold inductions/repressions	
	Based on concentrations in µM	
	Phenylethanol	Tyrosol
Aromatic amino acid (50 µM/ml)		
Phenylalanine	22 ± 2.3	2.7 ± 0.60
Tyrosine	1.5 ± 0.18	87 ± 15
Tryptophan	2.4 ± 0.75	5.3 ± 0.10
Ammonium sulfate		
50mM	0.78 ± 0.011	nd
100mM	0.82 ± 0.23	nd
500mM	0.77 ± 0.069	0.46 ± 0.00088
1000mM	0.86 ± 0.075	1.7 ± 0.28
5000mM	0.63 ± 0.11	0.30 ± 0.16
37000mM	0.63 ± 0.015	0.32 ± 0.042
Sodium chloride		
2% (w/v)	0.59 ± 0.0035	0.63 ± 0.064
4% (w/v)	0.74 ± 0.067	0.66 ± 0.14
6% (w/v)	0.51 ± 0.015	0.48 ± 0.097
8% (w/v)	0.34 ± 0.047	0.23 ± 0.0051
pH		
5.0	0.80 ± 0.063	0.80 ± 0.0050
6.0	0.87 ± 0.27	1.8 ± 0.15
7.0	nd	0.31 ± 0.11
8.0	nd	nd
9.0	nd	nd
Temperature		
15°C	0.85 ± 0.55	0.81 ± 0.43
Aromatic alcohol (50 µM/ml)		
Phenylethanol	-	3.8 ± 0.38
Tyrosol	1.2 ± 0.18	-
Tryptophol	1.2 ± 0.20	5.0 ± 0.94

nd not detectable

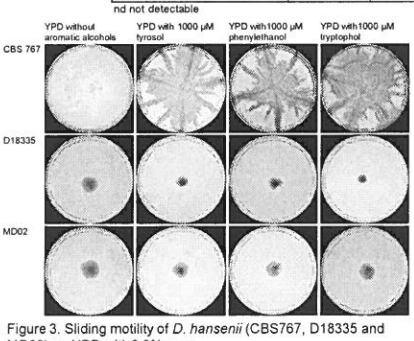

Figure 3. Sliding motility of *D. hansenii* (CBS767, D18335 and MD02) on YPD with 0.3% agar.

Figure 4. Pseudohyphal formation of *D. hansenii* (MD02) on low-ammonum agar plates.

Production of alcohols at standard conditions

D. hansenii (CBS 767, D18335 and MD02) were found to produce increasing concentrations of phenylethanol and tyrosol in the µM range during growth at standard conditions (Figure 1A). After 120 h of fermentation, the concentrations of phenylethanol and tyrosol were found to be 9.77-12.6 µM and 3.01-8.42 µM, respectively. Concerning tryptophol, this alcohol was in general not detected at standard conditions. For comparison, the concentrations of phenylethanol, tyrosol and tryptophol were 293, 119 and 11.4 µM for *C. albicans* (CBS 8758) and 31.4, 22.8 and 0.198 µM for *S. cerevisiae* (1171) (results not shown). The fact that alcohols were primarily determined from the end of exponential phase indicates that these alcohols are potential quorum sensing molecules in *D. hansenii*.

When calculated as µg/g dry wt, *D. hansenii* (CBS 767 and D18335) produced increasing concentrations of phenylethanol and tyrosol, whereas *D. hansenii* (MD02) produced constant concentrations of the two alcohols (Figure 1B). The fact that *D. hansenii* (CBS 767 and D18335) produced increasing concentrations of phenylethanol and tyrosol during stationary phase indicates that these alcohols besides being produced as potential quorum sensing molecules also are products used for survival of these two *D. hansenii* strains.

Alcohol production at different environmental conditions

The aromatic alcohol production for the three *D. hansenii* strains varied with different environmental conditions. Table 1 shows the changes in alcohol production at different environmental conditions for *D. hansenii* (CBS767) after 120 h of fermentation. Similar results were obtained for *D. hansenii* (D18335 and MD02) (results not shown). Addition of aromatic amino acids (the precursors of aromatic alcohol production) increased aromatic alcohol production in abundance. Phenylethanol production increased 22-fold by addition of phenylalanine, whereas tyrosol production increased 87-fold by the addition of tyrosine. Addition of tryptophane resulted in tryptophol concentrations between 4.20-7.50 µM. Furthermore, alcohols were not only increased by their own precursors e.g. tryptophane increased tyrosol production by more than 5.0-fold. Production of phenylethanol and tyrosol was generally decreased by increasing concentrations of ammonium, NaCl, pH and temperature values. Contrary to phenylethanol, tyrosol production were found to be autosimulated by addition of phenylethanol and tryptophol, as these alcohols increased tyrosol production by more than 3.8-fold.

Biofilm formation, sliding motility and pseudohyphal growth

Among *D. hansenii* strains investigated, especially *D. hansenii* (CBS767) was capable of biofilm formation (Figure 2a). Phenylethanol, tyrosol and tryptophol did in general not increase biofilm formation of *D. hansenii* (CBS767), whereas farnesol concentrations as low as 500 µM completely inhibited *D. hansenii* (CBS767) in formation of biofilm (Figure 2b). Also concerning sliding motility, *D. hansenii* (CBS767) shown a particular ability as this strain overgrew YPD plates with low agar content (Figure 3). The correlation between biofilm formation and sliding motility suggests an intimate connection of the two phenotypes. Addition of phenylethanol, tyrosol and tryptophol as low as 1000 µM resulted in more filamentous growth pattern *D. hansenii* (CBS767) (Figure 3). Contrary, only *D. hansenii* (MD02) was able of pseudohyphal growth on low-ammonium agar plates (Figure 4).

Conclusions

The present study shows that *D. hansenii* produces alcohol-based quorum sensing molecules previously reported for *C. albicans* and *S. cerevisiae*. However, alcohol yields are highly dependent on growth conditions including the availability of aromatic amino acids, ammonium sulphate, NaCl, pH and temperature. Several physiological traits including biofilm formation and sliding motility of *D. hansenii* seems to be regulated by alcohol-based quorum sensing molecules. Knowledge on quorum sensing properties of dairy-relevant microorganisms including *D. hansenii* may be used in optimisation of the cheese ripening processes.

Acknowledgements

This work was supported by The Danish Dairy Research Foundation (Danish Dairy Board) and The Danish Ministry of Food, Agriculture and Fisheries. Janne Benjaminsen, Søren Nørgaard Kristiansen and Kenneth Hansen are thanked for excellent technical assistance.